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Feeding plasticity more than metabolic rate drives the productivity of economically important filter feeders in response to elevated CO_2 and reduced salinity

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Running Head: Effects of CO_2 and salinity on feeding and metabolism

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Abstract

Climate Change driven alterations in salinity and carbonate chemistry are predicted to have significant implications particularly for northern costal organisms, including the economically important filter feeders *Mytilus edulis* and *Ciona intestinalis.* However, despite a growing number of studies investigating the biological effects of multiple environmental stressors, the combined effects of elevated pCO_2 and reduced salinity remain comparatively understudied. Changes in metabolic costs associated with homeostasis and feeding/digestion in response to environmental stressors may reallocate energy from growth and reproduction, affecting performance. Although these energetic trade-offs in response to changes in routine metabolic rates have been well demonstrated fewer studies have investigated how these are affected by changes in feeding plasticity. Consequently, the present study investigated the combined effects of 26 days' exposure to elevated pCO_2 (500 µatm and 1000 µatm) and reduced salinity (30, 23 and 16) on the energy available for growth and performance (Scope for Growth) in *M. edulis* and *C. intestinalis*, and the role of metabolic rate (oxygen uptake) and feeding plasticity (clearance rate and absorption efficiency) in this process. In *M. edulis* exposure to elevated pCO_2 resulted in a 50% reduction in Scope for Growth. However, elevated pCO_2 had a much greater effect on *C. intestinalis*, with more than a 70% reduction in Scope for Growth. In *M. edulis* negative responses to elevated pCO_2 are also unlikely be further affected by changes in salinity between 16 and 30. Whereas, under future predicted levels of pCO_2 *C. intestinalis* showed 100% mortality at a salinity of 16, and a >90% decrease in Scope for Growth with reduced biomass at a salinity of 23. Importantly, this work demonstrates energy available for production is more dependent on feeding plasticity, i.e. the ability to regulate clearance rate and absorption efficiency, in response to multiple stressors than on more commonly studied changes in metabolic rates.

Introduction

Climate change is leading to simultaneous alterations in several environmental factors including ocean temperature, pH and salinity (e.g. Doney *et al*. 2009). Rising levels of CO_2 in the atmosphere are causing increases in sea surface temperature, and worldwide modification of ocean carbonate chemistry, with gradual reductions in pH and carbonate ion (CO_3^{2-}) availability in a process known as ocean acidification (e.g. Sabine and Feely 2007; Doney *et al*. 2009). Elevated atmospheric CO_2 and associated temperature changes are also affecting weather patterns and are altering the Earth's hydrological cycle, which in turn, affects ocean salinity (Pierce *et al*. 2012). Freshening of surface salinity has been occurring over past decades with some of the largest reductions in salinity taking place at higher latitudes because of increased precipitation, freshwater runoff, melting freshwater ice and alterations in the meridional overturning circulation (Callaghan *et al*. 2011). Most studies to date on the effects of elevated pCO_2 and reduced salinity have focused on their individual effects. Recently, however, the combined effects of various factors have received some attention, as these may differ from the examination of each factor individually (e.g. Harvey *et al*. 2013; ; Sokolova *et al.* 2016).

Reduced salinity is a prominent stress factor in Arctic and Subarctic coastal areas with future changes predicted to have significant implications for northern estuarine and fjord ecosystems (e.g. Biggs and Cronin 1981; Callaghan *et al*. 2011). Such species include economically important filter feeders such as the blue mussel *Mytilus edulis* and the invasive ascidian *Ciona intestinalis* (Locke and Carman 2009). Colder higher latitude waters also absorb more CO_2 than warmer waters resulting in a greater pH change and lower levels of calcium carbonate saturation at a given pCO_2 level (Takahashi *et al*. 2014). The effect of elevated pCO_2 on seawater pH may also be increased in these areas as reduced salinity will reduce the total alkalinity and buffering capacity of seawater (Lee *et al*., 2006). Tolerances to elevated pCO_{22} vary among marine invertebrate species, as do tolerances to changes in salinity (e.g. Sokolova *et al.* 2016; Wood *et al.* 2016). Little, however, is known about their combined effects and our current understanding in filter feeders is limited to just a few studies where it has been shown that these factors influence the survival, energy metabolism and osmoregulatory capacity as well as weaken shells. (e.g. Dickinson *et al.* 2013; Velez *et al*. 2016). It is possible that the tolerance of *M. edulis* and *C. intestinalis* to the combined effects of elevated pCO_2 and reduced salinity may differ. If so, this could potentially

affect community structure via alterations in competitive interactions between the two species, which are known to have an economic impact on *M. edulis* aquaculture (e.g. Locke and Carman 2009).

Although *M. edulis* and *C. intestinalis* have long been considered osmoconformers with little extracellular ionic control (Shumway 1977; 1978)) both species have adapted/acclimatised to wide natural gradients in salinity. For example, in the Baltic Sea the lowest salinity limit for development in *C. intestinalis* is as low as 11 (Dybern 1967), and the natural distribution of *M. edulis* is only limited by salinities lower than 4.5 (Segerstråle 1944). However, laboratory studies suggest that optimum fertilisation and early development of *C. intestinalis* occurs above 34 salinity, with much wider tolerance ranges for pH, between 7.4 and 8.8 (Ballas *et al*. 2003). In *M. edulis* adaptation/acclimatisation appears to come at an energetic cost with low salinity populations exhibiting reductions in growth, longevity and reproductive fitness (e.g. Westerbom *et al*. 2002), with metabolic rates (i.e. the cost of living) increase linearly between 30 to 10 salinity (Stickle and Sabourin 1979). However, metabolic responses to reduced salinity are dependent on ion-regulatory capacity, with euryhaline invertebrates demonstrating increased metabolic rates when exposed to reduced salinity and stenohaline invertebrates, such as *M. edulis* and *C. intestinalis*, demonstrating decreased metabolic rates (Shumway 1978). Calcification in *M. edulis* is limited by low salinity (lower salinity threshold for calcification between 14.7 and 20; Malone & Dodd 1967), as well as elevated pCO_2 (e.g. Fitzer *et al*. 2016) possibly affecting the overall cost of calcification and so growth. Under elevated pCO_2, increased cellular energy demands limit energy available for growth and productivity (Thomsen and Melzner 2010), although food availability and feeding rate are determining factors (Thomsen *et al*. 2013). Both elevated pCO_2 and reduced salinity can affect energetic demand and resource allocation affecting performance, productivity and survival. However, changes (both positive or negative) in energy absorption via feeding, which in filter feeders is dependent on clearance rate and absorption efficiency, are likely as important in determining energy budgets as changes in metabolic rate. Metabolic costs changing directly with feeding due to specific dynamic action (Gaffney and Diehl 1986; Sigsgaard *et al*. 2003). Despite the importance of feeding plasticity in determining energy availability for growth and performance, little is known of how responses, such as clearance rate and absorption efficiency, interact to affect overall energy absorption in filter feeders when challenged by elevated pCO_2 and/or reduced salinity. In general,

filter feeders reduce pumping rates in response to reduced salinity, linked to decreased clearance rates (e.g. Anderson and Prosser 1953; Shumway 1977; Shumway 1978). However, clearance rates and particle retention are much more plastic that previously supposed (e.g. Denis *et al*. 1999; Strohmeier *et al*. 2009; Strohmeier *et al*. 2012; Cranford *et al*. 2016), with some bivalves up regulating clearance rates at times of energy limitation (Denis *et al*. 1999). In addition to clearance rate, absorption efficiency of digestion is also an important determinant of overall energy absorption through feeding, which also shows plasticity. In the Atlantic Deep Sea Scallop (*Palctopecten magellanicus*), for example, mean absorption efficiency has been shown to increase as filtration rates decreased in an attempt to maintain total energy absorption through feeding (e.g. Cranford and Hargrave 1994). To date, the effects of elevated pCO_2 on the absorption efficiency of marine organisms is not well understood (Navarro *et al*. 2013). Some species, for example *Mytilus chilensis*, reduce absorption efficiency (Navarro *et al*., 2013) and others such as the Mediterranean Mussel (*Mytilus galloprovincialis*) increase absorption efficiency in response to elevated pCO_2 (Fernandez-Reiriz *et al*. 2012). Four-week exposure to reduced salinities in the mussel, *Perna viridis*, resulted in reduced absorption efficiency (Wang *et al*. 2011).

The role of feeding plasticity in determining energy budgets is poorly understood. This study investigates the combined effects of elevated pCO_2 and reduced salinity on the energy available for growth and performance in *M. edulis* and *C. intestinalis*, and the role of feeding plasticity in this process. Both *M. edulis* and *C. intestinalis* were exposed to chronic mid-term (26 days) elevated pCO_2 and reduced salinity. At the end of the exposure period, oxygen uptake rates were determined as a proxy for routine metabolic rates, and clearance rate and absorption efficiency were determined to assess the ability to exploit feeding plasticity. Fitness/performance was examined by measuring growth and mortality rates. Experiments were used to assess which species would be more likely to survive near future conditions of increasing pCO_2 and declining salinity due to occur along northern costs.

Materials and Methods

Animal Collection and acclimation

Adult *C. intestinalis* (3.8±0.1 g FW, 5,0±0,5 cm length) and *M. edulis* (16.3±0.6 g FW,

5.0±0.06 cm length) were collected from the shallow subtidal zone at the Institute of Marine Research, Austevoll, Norway (60°05'08.9"N, 05°15'42.5"E) in November 2015. Ninety *C. intestinalis* and forty *M. edulis* were weighed as a baseline to monitor growth. The animals were then glued to pieces of velcro in preparation for attachment to the sides of the experimental tanks, mimicking their natural hanging position. The animals were left to recover for 48 h in aerated ambient seawater prior to acclimation to experimental conditions. Five *C. intestinalis* and three *M. edulis* were assigned to each treatment tank (4 L) and the tanks were triplicated per experimental treatment (N = 15 *C. intestinalis*; N= 9 *M. edulis* per treatment). After being assigned to treatment tanks, salinity and pCO_2 levels were changed from ambient to the final treatment conditions over approximately 6 h.

The treatments consisted of three salinity levels (30, 23 and 16) and two pCO_2 levels (500 and 1000 µatm) in a fully crossed design. Treatments were maintained using a flow-through system, using unfiltered seawater pumped (7m depth), directly from the site of animal collection and supplied to each treatment tank at a flow ≈ 50 L h^{-1}. This insured that control treatments corresponded to natural pCO_2 and salinity levels. Seawater salinity levels for each experimental treatment were maintained by mixing with un-chlorinated freshwater (source, Vannområde Vest Austevoll), before being supplied to 6 header tanks (1 per treatment) where pCO_2 levels were controlled. A nominal control pCO_2 value of 500 µatm was selected as this corresponded to the natural habitat pCO_2 level that the organisms were acclimatised to at the time of collection. Carbonate chemistry in Norwegian fjords is extremely dynamic and elevated pCO_2 levels compared to the open ocean can be associated with seasonal decreases in primary productivity (e.g. Fransson et al 2016). To achieve a predicted future elevated pCO_2 level of 1000 µatm (e.g. Caldeira and Wickett 2003) the pH was individually controlled for each salinity treatment taking into account the effect of temperature, salinity and total alkalinity (30 = pH 7.676; 23 = pH 7.598; 16 = pH 7.489) calculated using free-access CO_2SYS (Lewis and Wallace 1998). CO_2 levels were achieved by the addition of elevated PCO_2 seawater (pH 5.5) to each header tank via peristaltic pumps controlled according to seawater pH levels via pH electrodes connected a controller (Endress and Hauser, Liquiline CM448; after, Andersen *et al*., 2013). The flow through system was placed within a temperature controlled room to maintain at 10°C throughout the experiment. Salinity, pH and temperature in each individual tank were

recorded three times a day using a handheld multimeter (labquest 2, vernier). Total alkalinity (TA) was measured twice a week by titration (TIM840 titration manager, TitraLab). Values for the physicochemical parameters and the associated carbonate chemistry values for this system are presented in Table 1. Following 26 days of acclimation a number of responses were determined in order to assess energy allocation in *C. intestinalis* and *M. edulis* as a result of combined exposure to elevated pCO_2 and reduced salinity.

Determination of feeding rate and energy absorption

Particle clearance rate (CR) of 9 *C. intestinalis* and 9 *M. edulis* was determined as an estimate of feeding rate and for the calculation of energy ingestion using the flow through feeding chambers developed by Strohmeier *et al*. (2009). These chambers were supplied with the same unfiltered seawater as the animals in the respective treatment tanks. Three chambers were left empty as controls. Internal dimensions of the *C. intestinalis* feeding chambers were: width 5 x length 22 x height 10 (cm) and the *M. edulis* chambers were: width 3.8 x length 19.5 x height 8 (cm). These chambers have been demonstrated to restrict recirculation and therefore inhibit the animals from re-filtering the water (Strohmeier *et al*. 2009; Cranford *et al*. 2016). The rate of water flow was maintained to a level that would also ensure no re-filtration (nominal set values; *M. edulis* = 10 l h^{-1}; *C. intestinalis* = 6 l h^{-1}). The animals were placed in the chambers and allowed to rest for 1h undisturbed to resume feeding behaviour prior to sampling before the concentration of suspended particles (within 30 size-interval between 1 and 60µm in diameter) in the out-flow seawater from each chamber was measured using a laser particle counter (PAMAS GmbH, Model S4031GO). This protocol was repeated 3 times on the same 9 individuals and 3 controls from each respective species and treatment. As each of the 6 treatments were repeated 3 times, 6 hours apart, and each feeding trial took just over 1 h (1h of resting time, a few minutes to collect the water and then change the treatment) clearance rate and POM data was collected over a 12 hour period before the faecal collection as describe below. Therefore, the food in the gut that was defecated during faecal collection was cleared by the animal during the feeding trials. CR (l h^{-1}) was then calculated using the equation:

$$CR = F(C_{in}-C_{out})/C_{in} \quad (1)$$

Where F (l h^{-1}) is the measured flow rate of water though each individual chamber. C_{in} is the inflow concentration of food represented by the particle concentration from the control chambers, and C_{out} is the partial concentration from each experimental chamber.

To calculate energy ingestion from CR particulate organic matter (POM) was determined for each feeding experiment. POM was determined by collecting 4 L of seawater from each of the 3 control chambers and filtering through pre- combusted (450 ° C for 5 hours to remove carbon) and pre-weighed 1.5 µm glass microfiber filters (VWR) using 1 ml of ammonium formate to remove salt crystals. Filters were dried to determine dry weight (DW; 60°C for 24 hours) and ash free-dry weight (AFDW; 450°C for 5 hours) to establish organic content of the POM. This protocol was repeated three times for each control chamber. Energy ingested through feeding was then estimated by multiplying CR by the concentration of POM (mg AFDW L^{-1}) and by the energetic content of POM (23 J mg AFDW $^{-1}$; Widdows *et al*. 1979).

Following feeding experiments the animals were placed in individual chambers constructed from sections of PVC pipe (length 12 cm, diameter 8cm) with mesh attached to each end (diameter 375 µm). After 24 h any faecal pellets in the chambers were filtered onto pre-weighed and burned filters (described above) using distilled water. Following this the filters were dried to determine DW (60°C for 24 hours) and AFDW (450°C for 5 hours) to establish organic content of the faecal pellets. Absorption efficiency was then estimated from the ratio of the organic content of the seston (POM) averaged over the 12h feeding period prior to faecal collection and the organic content of the faeces, using the equation (after, Conover 1966):

$$\text{Absorption Efficiency} = (F-E) / ((1-E)F) \quad (2)$$

Where F is the ash-free dry weight: dry weight ratio of the seston during feeding and E is the ash-free dry weight: dry weight ratio of the faeces. Energy absorption though feeding was then estimated by multiplying the energy ingested by the absorption efficiency.

Rates of oxygen uptake

Oxygen uptake rate was measured as a proxy for metabolic rate ($\dot{M}O_2$). $\dot{M}O_2$ was measured using stop-flow respirometry after Garilli *et al*. (2015) and Harvey *et al*.

(2016). In brief, individual C. *intestinalis* and *M. edulis* from each treatment were placed in individual chambers (volume 160 ml) supplied with the same seawater as the respective treatments tanks (flow rate ≈ 10 L h^{-1}). Animals were allowed 1 h to recover from handling and regain natural ventilatory behaviour before the flow to each chamber was closed and the decreases in % oxygen saturation continuously measured using a non-invasive optical oxygen system (Oxy-10 mini, PreSense; labquest 2, Vernier) modified from Rastrick and Whiteley (2011) and Calosi *et al*., (2013). The incubation period was 5 h for *C. intestinalis* and 3 h for *M. edulis*, during which time, % oxygen saturation levels of the seawater did not fall below 70% to avoid hypoxic conditions. A blank chamber with no animal was monitored in parallel to each treatment to account for background respiration in the seawater. Percentage oxygen saturation was converted to oxygen partial pressure (P_{O_2}) adjusted for atmospheric pressure and vapour pressure adjusted for relative humidity (continuously monitored using a multimeter; Labquest 2, Vernier). $\dot{M}O_2$ was calculated from the decrease in P_{O_2} within each chamber multiplied by the oxygen solubility of seawater using coefficients adjusted for the effect of temperature and salinity (Benson and Krause, 1984), and expressed as μmol O_2 h^{-1}. $\dot{M}O_2$ was then used to estimate the amount of absorbed energy lost via metabolism (routine metabolic maintenance of homeostasis, feeding and digestion) assuming a heat equivalent of oxygen uptake of 0.456 j μmol^{-1} O_2 (Gnaiger, 1983).

Growth

Estimates of energy availability for growth and reproduction (Scope for Growth; SfG) for each treatment were calculated from estimates of rates of energy absorption though feeding (EA; j h^{-1}) and energy loss via metabolism (EL; j h^{-1}; modified from Widdows and Johnson 1988):

$$SfG\ (j\ h^{-1}) = EA - EL \quad (3)$$

During the incubation period the wet weight (g) of each animal was recorded twice a week to determine growth rates. At the end of the acclimation the soft tissue of *C. intestinalis* and *M. edulis* individuals was dried to determine dry weight (60°C for 48 hours) and ash free-dry weight (450°C for 5 hours) to establish any treatment effects on carbon richness (energy density) of the tissue.

Statistical Analysis

The effects of elevated pCO_2 and/or reduced salinity (fixed factors) on all of the measured parameters (dependent factors) were tested using a nested general linear mixed model (GLMM) with body mass as a covariate (to adjusted for the effect of variation in body size between individuals) and tank as a random factor nested within the fixed factors. This considers that replicate tanks were supplied by a single header tank per treatment and therefore, despite being a flow through system, tanks may not be considered true replicates (e.g. Collard *et al*., 2015; Small *et al*., 2015). Any observed significant differences were further analysed by F-tests based on pairwise comparisons generated from the estimated marginal means of the GLMM. Proportional data was arc sign square root transformed before statistical analysis. All values are expressed as means ± SEM. All statistical analyses were performed using SPS software (v 20 SPS Chicago, Ill, USA).

Results

Mortality

After 20 days of the 26-day exposure period *C. intestinalis* showed 0% survivorship in the lowest salinity of 16. Consequently, further energetic parameters could not be determined in this treatment. After 26-days the lowest survivorship of 53% was recorded in the 23 salinity and elevated pCO_2 treatment, followed by 80% in the ambient salinity of 30 and elevated pCO_2 treatment. At ambient PCO_2, 87% and 90% survivorship was reported for salinities of 23 and 30, respectively. Conversely over the 26-day exposure period only one mortality was reported for *M. edulis* across all treatments.

Feeding - clearance rate and energy ingestion

In *C. intestinalis* elevated pCO_2 significantly influenced the effect of salinity on CR ($F_{1,29}$=10.291, P= 0.003) and energy ingestion ($F_{1,29}$= 8.938, P<0.01). In the ambient pCO_2 treatments, CR and energy ingestion were maintained across the salinity treatments. However, in the elevated pCO_2 treatments a reduction in salinity from 30 to 23 resulted in a significant reduction in CR in *C. intestinalis* from 0.9±0.1 L h^{-1} to 0.3 ±0.1 L h^{-1}, respectively ($F_{1,29}$ = 13.829, P<0.001; Figure 1C). This was associated with a significant decrease in energy ingestion between the same treatments ($F_{1,29}$=11.940, P<0.01; Table 2). Energy ingestion was also significantly lower in

elevated pCO_2 treatments at both a salinity of 30 ($F_{1,29}$ = 11,940, P<0.01) and 23 ($F_{1,29}$ = 62.765, P<0.001; Table 2).

In *M. edulis*, elevated pCO_2 significantly influenced the effect of salinity on CR ($F_{2,45}$ = 11.421, P<0.001; Figure 2, A) and energy ingestion ($F_{2,45}$ = 7.075, P<0.01; Table 3). In the ambient pCO_2 treatments, a reduction in salinity from 30 to 16 resulted in a significant reduction in CR from 2.5±0.2L h^{-1} to 1.0±0.2 L h^{-1}, ($F_{2,45}$= 13.748, P<0.001; Figure 1D) and energy ingestion ($F_{2,45}$=31.167, P<0.001; Table 3), respectively. However, in the elevated pCO_2 treatments, CR was maintained across the salinity treatments, driving the interaction. Overall in *M. edulis*, elevated pCO_2 resulted in an increase in CR ($F_{1,45}$ = 62.555, P<0.001) and energy ingestion ($F_{1,45}$ = 5.640, P<0.05). This was driven by significantly higher CR in the combined elevated pCO_2 and reduced salinity treatments (23 salinity, $F_{1,45}$ = 13.345, P<0.001; 16 salinity, $F_{1,45}$ = 70.185, P<0.001).

Feeding - Absorption Efficiency

The absorption efficiency of surviving *C. intestinalis* showed no significant variation between salinity treatments of 30 and 23 ($F_{1,26}$=0.065, P=0.801) or pCO_2 combinations ($F_{1,4}$=4.730, P=0.099; Table 2). *M. edulis* showed an increase in absorption efficiency at reduced salinity, although this pattern was significantly influenced by pCO_2 ($F_{2,39}$ = 7.296, P<0.05; Table 3). In the ambient pCO_2 treatments, salinity had a greater effect on absorption efficiency with significantly higher absorption efficiencies at salinities of both 23 and 16 compared with ambient salinity ($F_{2,12}$ = 20.443, P<0.001; Table 3). At elevated pCO_2, the effects of salinity were weaker and like *C. intestinalis* there was no significant difference in absorption efficiency between the ambient and 23 salinity treatments. Although, absorption efficiency did significantly increase at the lowest salinity of 16 compared with ambient salinity ($F_{2,12}$ = 4.304, P<0.05; Table 3). This interaction was, in part, driven by a significant reduction in absorption efficiency at elevated PCO_2 across all salinity treatments ($F_{2,39}$ = 7.296, P<0.05).

Feeding - Energy Absorption

The energy absorption of *C. intestinalis* estimated from energy ingested through feeding and absorption efficiency showed no significant variation between those surviving salinity treatments at ambient or elevated pCO_2 levels ($F_{1,29}$ = 0.508, P=0.482). However, energy absorption was lower in *C. intestinalis* at elevated

compared with ambient pCO_2 levels, with significant reductions of 66% ($F_{1,29}$= 8.378, P>0.01) and 93% ($F_{1,29}$= 19.287, P>0.001) in the 30 and 23 salinity treatments, respectively.

In *M. edulis* the effect of salinity on energy absorption was significantly influenced by pCO_2 levels ($F_{2,41}$ = 18.930, P<0.001). At ambient pCO_2, energy absorption showed a slight but significant increase between ambient salinity and a salinity of the 23 (mean difference $_{2,41}$ = 4.952±2.398, P<0.05). However, at the lower salinity of 16, energy absorption significantly decreased compared to ambient salinity, to levels similar to those reported across the elevated pCO_2 treatments (mean difference $_{2,41}$ = -12.465±2.393, P<0.001). In the elevated pCO_2 treatments, energy absorption was significantly lower than the values at ambient pCO_2 across all salinities ($F_{1,4}$=17.360, P<0.05; Table 3) and showed no significant variation with salinity ($F_{2,41}$=1.265, P=0.293; Table 3).

Metabolic Rate

In *C. intestinalis*, rates of oxygen uptake ($\dot{M}O_2$) were significantly lower at a salinity of 23 compared to the ambient salinity at both pCO_2 levels ($F_{1,4}$ = 146.901, P<0.001; Figure 1A). However, in the same species $\dot{M}O_2$ were significantly higher in the elevated compared to the ambient pCO_2 treatments at both the 23 and the ambient salinity treatments ($F_{1,25}$ = 35.701, P<0.001; Figure 1A).

In *M. edulis* $\dot{M}O_2$ was also significantly lower at reduced salinity compared with ambient treatments, but only at the elevated pCO_2 levels. (23 salinity, $F_{7,51}$=5.154, P<0.05; 16 salinity, $F_{7,51}$=4.980, P<0.05; Figure 1B). In contrast and similar to C. *intestinalis, M. edulis* exhibited a significant increase in MO_2 at elevated pCO_2 across all salinity treatments ($F_{1,3}$ = 38.089, P<0.01; Figure 1B). In *M. edulis*, $\dot{M}O_2$ also decreased significantly in association with a decrease in CR (Spearmen Rank, correlation coefficient$_{58}$ = 0.426, p<0.01).

Growth

In *C. intestinalis* estimated energy available for growth and reproduction (SfG) showed no variation among salinity treatments at the ambient ($F_{1,29}$=0.022, P=0.884) or elevated pCO_2 treatments ($F_{1,29}$=1.438, P=0.240). Despite conservation of SfG across the 23 and ambient salinity treatments at ambient pCO_2 levels, growth rate significantly decreased from 0.035±0.013 g day^{-1} at ambient salinity to -0.007 g day^{-1} and -0.011 g

401 day^{-1} at salinities of 23 and 16, respectively ($F_{2,68}$=3.521, P<0.05). Negative growth at
402 a salinity of 23 was accompanied by a significant increase in AFDW: DW ratio at
403 ambient pCO_2 ($F_{1,8}$=18.396, P<0.01; Table 2). However, at elevated pCO_2 levels,
404 growth rate was unaffected by a change in salinity from 30 to 23 ($F_{2,68}$=0.692, P=0.504).
405 Despite no changes in SfG between a salinity of 30 and 23 at either pCO_2 level,
406 there were significant decrease in SfG in the elevated compared to the ambient pCO_2
407 treatments at both salinities ($F_{1,29}$=226.690, P<0.001). In the ambient salinity treatment,
408 SfG was reduced by more than 70% at elevated compared with ambient pCO_2 levels
409 ($F_{1,29}$=8.468, P<0.01). This was associated with a significant reduction in AFDW:DW
410 ratio of the tissues ($F_{1,8}$=7.414, P<0.05). However, due to large variations between
411 individuals, this was not associated with a significant reduction in growth rate
412 ($F_{1,68}$=0.605, P=0.439). At a reduced salinity of 23, elevated pCO_2 had a greater effect
413 on SfG than at ambient salinities, with more than a 90% decrease between ambient and
414 elevated pCO_2 treatments ($F_{1,29}$=19.360, P<0.001).
415 Overall, in *M. edulis*, SfG was significantly lower in the elevated pCO_2
416 treatments compared with ambient pCO_2 levels ($F_{1,4}$=19.162, P<0.05). In addition, the
417 effect of salinity on SfG was significantly influenced by elevated PCO_2 ($F_{2,40}$=18.367,
418 P<0.001). At ambient pCO_2, *M. edulis* showed a small but significant increase in SfG
419 between ambient and the 23 salinity treatments (mean difference $_{2,40}$= 5.096±2.422,
420 P<0.05; Table 3), but a significant decrease at the lowest salinity of 16 (mean difference
421 $_{2,40}$= -12.352±2.418, P<0.001). However, there was no significant variation in SfG
422 between salinity treatments at elevated pCO_2 ($F_{2,41}$=1.641, P=0.206). Patterns in SfG
423 were reflected in observed growth rate. At ambient pCO_2, growth rate was maintained
424 unchanged between ambient and the 23 salinity treatment (mean difference $_{2,39}$= -
425 0.01±0.008, P=0.227), but significantly decreased from 0.035±0.006 g day^{-1} in the
426 ambient salinity treatment to 0.014 g day^{-1} in the 16 salinity treatment (mean difference
427 $_{2,39}$= -0.021±0.008, P<0.05). Growth rates did not vary significantly between salinity
428 treatments at elevated pCO_2 levels ($F_{2,39}$=0.754, P=0.477). AFDW: DW ratios showed
429 no significant variation between PCO_2 ($F_{1,2}$=11.458, P=0.082) or salinity treatments
430 ($F_{2,5}$=0.442, P=0.668; Table 3).
431
432 **Discussion**
433 *Feeding responses to combined elevated pCO_2 and reduced salinity*
434 Following 26 days exposure to the combined treatment, surviving tunicates maintained

CR and energy absorption between salinities of 30 and 23 at present ambient levels of pCO_2. However, pCO_2 levels associated with predicted OA had a synergistic effect with the lowest CR recorded in the elevated pCO_2 and reduced salinity treatment. As there was no significant difference in POM between treatments, energy ingestion was also lowest under elevated pCO_2 and reduced salinity. Reductions in CR may result from reduced pumping activity and siphon retraction. *C. intestinalis* have a single inhalant siphon which they utilise for feeding and respiration. During repeated short-term exposure to a reduced salinity of 19, *C. intestinalis* close their siphons to avoid internal exposure to low salinity seawater, thereby avoiding osmotic imbalance (Shumway, 1978). Pumping rates remained reduced until external salinity levels were restored to normal (Shumway, 1978). As *C. intestinalis* exhibited no significant variation in absorption efficiency across experimental treatments, reduced energy ingestion resulted in an uncompensated decrease in total energy absorption.

In the ambient pCO_2 treatments, *M. edulis* demonstrated a reduction in CR in the lowest salinity treatment (16). *M. edulis* has also been shown to exhibit reduced pumping activity in order to limit internal exposure to low salinity water (Shumway 1977). Both species show little ionic- or osmo-regulatory capacity, they have developed this response to isolate the tissues from exposure to reduced salinity conditions and associated ionic stress. Valve closure in response to decreases (50%) in sea water concentration has previously been reported in hard clam (*Mercenaria mercenaria*; Anderson & Prosser 1953) and the pacific oyster (*Crassostrea gigas*; Shumway 1977). Longer-term (4 week) exposure to reduced salinity levels comparable with the present study also led to reduced CR in the mussel, *Perna viridis* (Wang *et al*. 2011). Here reduced salinity did not result in a complete loss of pumping activity, that would restrict gas exchange, but reduced CR are likely to be involved with this general strategy to limited internal exposure to reduced salinities.

In contrast to *C. intestinalis, M. edulis* do partially compensate for reduced CR by up regulating absorption efficiency at lower salinities. The Atlantic Deep Sea Scallop (*Placopecten magellanicus*) also increases absorption efficiency as filtration rates decreased (e.g. Cranford & Hargrave 1994; *cf*, Wang *et al*. 2011). At ambient pCO_2 this up regulation in absorption efficiency in the 23 salinity treatment leads to a slight but significant increase in overall energy absorption. However, at a salinity of 16 this compensation is incomplete resulting in lower overall energy absorption.

Compensatory changes in absorption efficiency may be limited due to an overall reduction in absorption efficiency at elevated pCO_2, as also shown for Juvenile *Mytilus chilensis* (Navarro *et al*. 2013). Sea urchin larvae (*Strongylocentrotus droebachiensis*) exposed to elevated pCO_2 also showed reduced digestion rates and a 0.3-0.5 pH unit decrease in gut alkalinity, which was associated with decreased *in vitro* protease activity. Interestingly this pCO_2 induced reduction in digestive activity was partly compensated by increased feeding rates (Stumpp *et al*. 2013) as seen here.

In the present study, reduced absorption efficiency reported in the elevated pCO_2 treatments may also change the acclimatory strategy of *M. edulis* to reduced salinity. In contrast to ambient pCO_2 treatments where CR are reduced at low salinities, at elevated pCO_2 CR are maintained across all salinities, possibly to compensate for the reduction in absorption efficiency. CR and particle retention efficiency in bivalves may be much more plastic than previously thought (e.g. Denis *et al*. 1999; Strohmeier *et al*. 2009, Strohmeier *et al*. 2012; Cranford *et al*. 2016). For example, *M. galloprovincialis* can increase CR maintaining energy absorption during food limitation (Denis *et al*. 1999). However, the maintenance of CR at lower salinity and elevated pCO_2 here is not sufficient to fully compensate for pCO_2-associated reductions in absorption efficiency, resulting in lower overall energy absorption. Relative increases in CR/pumping represents a trade-off between exposure of internal tissues to unfavourable conditions and the attempted maintenance of total energy absorbance though feeding, which is likely sensitive to the length of exposure and size of energy reserves. When exposed to elevated pCO_2, the maintenance of ventilation rates, associated with pumping activity, may also facilitate greater CO_2 excretion (e.g. Donohue *et al*., 2012)..

Metabolic responses to combined elevated PCO_2 and reduced salinity

In general *M. edulis* and *C. intestinalis* exhibited similar metabolic responses to combined pCO_2 and salinity conditions, with $\dot{M}O_2$ decreasing in response to reduced salinity and increasing in response to elevated pCO_2. Although in *M. edulis* significant decreases in $\dot{M}O_2$ at the 23 and 16 salinity treatments were limited to the elevated pCO_2 treatment. Several studies have reported increased metabolic rates in response to elevated pCO_2 in marine invertebrates (*e.g.* Wood *et al.* 2008; Beniash *et al.* 2010; Calosi *et al.* 2013) including *M. edulis* (Thomsen and Melzner 2010). Elevated metabolic rates may be associated with increased energetic costs of maintaining

physiological homeostasis (Wood *et al* 2008; Beniash *et al.* 2010). Conversely, reduced metabolic rate may help conserve energy at more extreme pCO_2/pH levels outside current predictions for ocean acidification (Langenbuch and Portner 2004); as shown by *M. galloprovinicali* following 3 months' exposure to pH 7.3 (Michaelidis *et al*. 2005) and *M. edulis* following 2 months' exposure to pH 7.14 (Thomsen and Melzner 2010). However, metabolic depression may not be sustainable in the longer-term with molluscs adapted/ acclimatised to naturally elevated pCO_2 ecosystems requiring elevated metabolic rates per gram of tissue to maintain performance (Harvey *et al*. 2016; Garilli, *et al*. 2015).

Metabolic responses to reduced salinity vary greatly in marine invertebrates with both increases (e.g. Navarro 1988) and decreases (e.g. Shumway 1978) in $\dot{M}O_2$ reported. In general, this response is dependent on ion-regulatory capacity, with stenohaline invertebrates, such as *M. edulis* and *C. intestinalis,* demonstrating a decrease in $\dot{M}O_2$ (Shumway 1978), as reported here. *C. intestinalis* also reduces $\dot{M}O_2$ in response to decreased salinity, possibly associated with a reduction in ventilation and pumping activity (Shumway 1978). *M. edulis* and other bivalves also demonstrate reduced pumping to protect their internal structures from reduced salinity seawater (Anderson and Prosser 1953; Shumway 1977). Reduced pumping has been shown to reduce oxygen tension in the mantle cavity of *M. edulis* (Tang and Riisgård 2016) and the clam *Arctica islandica* (Taylor 1976). Valve control in *M. edulis* is postulated to regulate metabolic rate via reduction of oxygen partial pressure in the mantle cavity conserving energy during starvation (Tang and Riisgård 2016). However, as oxygen uptake is the result of metabolic demand for ATP and not an adaptive/acclimatory response, there is no known mechanism to explain how lowering oxygen availability via valve control could reduce oxygen demand without enzymatic feedback associated with harmful anaerobic pathways. This assumption also presumes that food limitation is ubiquitous with restricted valve opening and reduced CR, but this has been repeatedly questioned (e.g. Denis *et al*. 1999; Strohmeier *et al*. 2009; Strohmeier *et al*. 2012). Reduced activity due to restricted valve/siphon opening could reduce metabolic demand in response to low salinity (Shumway, 1978). Although, direct costs of pumping are estimated to be inconsequential in filter feeders (Jørgensen *et al*. 1986), reductions in metabolic rate due to reduced feeding and associated specific dynamic action (SDA) could be more significant. SDAaccounts for approximately 20% of oxygen uptake rates in both *M. edulis* and *C. intestinalis,* depending on food quality

(Gaffney and Diehl 1986; Sigsgaard *et al*. 2003). In a wide variety of filter feeders, including *M. edulis*, reduced feeding is associated with reduced metabolism (Thompson and Bayne 1972). Observed decreases in routine metabolic rate with decreased salinity here do not exclude the theoretical possibility that costs of maintaining homeostasis could increase, and that this ATP is reallocated from other energetically demanding processes such as feeding and digestion. As routine metabolic rates were determined in naturally fed animals no attempt is made to separate costs associated to maintaining homeostasis and costs associated with energy assimilation via feeding. This study instead focuses on the ecologically relevant overall energy requirement of the animal that, unlike some studies on starved animals, considers that natural levels of feeding and digestion have an energetic cost that may also lead to important trade-offs with growth and should therefore be included in a general assessment of energetic costs.

Resource allocation to growth

After 26 days incubation surviving tunicates showed a significant reduction in energy available for growth and reproduction (SfG) in the elevated pCO_2 treatments. This was a result of decreased energy absorption through feeding and to a lesser extent increased routine metabolic costs (including the costs of maintaining homeostasis and energy assimilation via feeding). In sea urchin larvae (*Strongylocentrotus purpuratus*) elevated pCO_2 (1271µatm); also reduced SfG, attributed to increased allocation of absorbed energy to metabolism. (Stumpp *et al*. 2011). Larvae in ambient pCO_2 conditions allocated between 78 and 80% of available energy to growth, whereas, larvae incubated at elevated pCO_2 invested only 39-45% (Stumpp *et al*., 2011). Increased costs of maintaining physiological homeostasis have also been postulated to reduce energy available for growth in the brittle star, *Amphiura filiformis*, exposed to simulated OA (Wood *et al*. 2008) and in gastropods inhabiting naturally elevated pCO_2 environments (Harvey *et al* 2015; Garilli *et al*., 2015). However, in the present study increased routine metabolic rates in *C. intestinalis* at elevated pCO_2 only accounted for a 1.2 j/day and 0.6 j/day decrease in SfG, at salinities of 30 and 23 respectively (calculated from differences in $\dot{M}O_2$ between pCO_2 treatments, Fig 1A, assuming a heat equivalent of oxygen uptake of 0.456 J µmol^{-1} O_2; Gnaiger 1983). Whereas, reductions in energy absorbed though feeding at elevated *p*CO had a much greater effect on SfG, reducing energy availability by 328.8 J day^{-1} at a salinity of 30 and 244.8 j day^{-1} at a salinity of 23 (calculated from differences in energy absorption between pCO_2

567 treatments, Table 2). Despite reductions in SfG, no significant reduction in growth rate
568 could be attributed to elevated pCO_2 at the ambient salinity, probably due to large
569 individual variability. However, patterns in SfG were consistent with patterns in
570 mortality among treatments. In treatments with surviving *C. intestinalis* after 26 days
571 the lowest SfG and highest mortality was observed at a salinity of 23 and elevated pCO_2
572 with the lowest mortalities observed in ambient pCO_2 treatments where SfG was
573 conserved between the ambient (30) and 23 salinity treatments.

574 Salinity caused mortality of 100% after 20 days in C. instestinalis similar to
575 other studies (e.g. Vercaemer et al 2011). In the 23 salinity and elevated pCO_2 treatment
576 *C. intestinalis* showed 53% mortality, here a selection may be possible as the survivors
577 that are examined are the most tolerant individuals within the population. Since
578 survivorship was above 80% in all other treatments, a selection effect is unlikely.
579 Growth rates also significantly decreased with salinity with a reduction in body mass
580 (negative growth) observed in all reduced salinity treatments. In the ambient pCO_2
581 treatment this reduction in body mass at reduced salinity occurred despite the
582 maintenance of SfG and survivorship, possibly attributable to a significant increase in
583 tissue AFDW:DW ratio. Consequently, in the 23 salinity treatment, available energy
584 (SfG) may be diverted toward storage and increased carbon richness of tissues at the
585 expense of overall growth. Although this is likely to increase the density of energy
586 stores the benefits of a reduction in body size are difficult to explain. Paleontological
587 and present reductions in body size, known as the Lilliput effect, associated with
588 adaptation to natural elevations in pCO_2 may, in part, help to maintaining metabolic
589 efficiency (Garilli *et al*. 2015). Although beyond the scope here, reduced body mass of
590 *C. intestinalis* in the ambient pCO_2 and reduced salinity treatment is associated with an
591 increase in mass specific metabolic rates while conserving whole animal energetic
592 demand compared to controls, possibly facilitating metabolic efficiency.
593 Across all treatments only one individual *M. edulis* died during the 26 day
594 incubation, in the ambient $pCO_2$and salinity treatment. Sessional variation in body mass
595 in Norwegian populations of *M. edulis* is highly dependent on reproduction, with body
596 mass increasing in early summer before decreasing with spawning between June and
597 August and then increasing again between September and December (e.g. Strohmeier
598 et al. 2015). Making late Autumn growth, as documented in the present study, important
599 to winter survival. The 8% increase recorded over 26 days under ambient conditions at

the same time of year is similar to previous studies (15-30% Strohmeier et al 2015)..

As in *C. intestinalis, M. edulis* showed a decrease in SfG with an elevation in pCO_2 in the ambient and 23 salinity treatments, attributable to reduced energy available through feeding and to a lesser extent an increase in metabolism. Despite less energy available for growth at elevated pCO_2, there was no significant effect of pCO_2 on growth rate within the 26 day period of exposure. Elevated pCO_2 can negatively affect the growth of *M. edulis* both under natural conditions in the Baltic Sea that resemble predicted OA (Thomsen and Melzner 2010; Thomsen *et al*., 2013), and in the laboratory (e.g. Fitzer *et al*. 2015), although pCO_2 levels and length of exposure varied.

At ambient pCO_2 levels SfG showed a slight but significant increase in the 23 salinity treatment compared to the controls, attributable to an elevation in absorption efficiency (discussed above), and results in the conservation of growth rates in this treatment. However, at the lowest salinity increased absorption efficiency cannot compensate for reduced CR and despite significant reduction in metabolic rates, SfG is reduced leading to a significant reduction in growth rate. Comparisons between populations of *M. edulis* from the North Sea and in the Baltic Sea where Baltic mussels living at comparatively lower salinities are frequently smaller than North Sea mussels, also attributed decreased growth to increased metabolic costs at lower salinities (Tedengren and Kautsky 1986) whereas in the present study decreases in SfG and associated growth rate at a salinity of 16 is due to reduced CR and not changes in metabolic rate. Interestingly salinity had no further effect on SfG or growth rates at elevated pCO_2 levels. Ammonia excretion only amounts to 1-2% of energy loss via metabolism during autumn (Bayne and Widdows 1978) and was therefore not considered here. In the high pCO_2 ambient salinity treatment where metabolic rates were highest ammonia excretion would amount to an estimated loss of 0.014-0.028 J h^{-1} which only represents 0.11-0.23% of the energy absorbed though feeding in this treatment and so any overestimation of SfG is considered inconsequential.

Conclusion and implications Under ambient salinities of 30 energy for mussel growth and reproduction could be reduced by up to 50% after mid-term exposure to elevated pCO_2 levels predicted for the end of the century, leading to possible losses for the aquaculture industry. However, growth rate of *C. intestinalis*, was reduced by 70% in energy for growth and reproduction under the same conditions possibly relieving pressure on the industry from this invasive tunicate. The reduction in SfG and growth

rate in mussels as a result of elevated pCO_2 is unlikely to be further affected by changes in salinity between 16 and 30. Whereas, under future predicted levels of pCO_2. *C. intestinalis* showed 100% mortality at a reduced salinity of 16 and showed more than 90% decrease in SfG with an associated mean reduction in biomass (negative growth) at a salinity of 23. Although future levels of ocean acidification may reduce mussel productivity, the effect on the industry may be, in part, compensated by the reduced productivity of invasive tunicates particularly during times of low salinity (e.g. seasonal precipitation or melt-water). Consequently, an elevated pCO_2 in future mussel aquaculture could also benefit from lower salinity sites. Although mid-term exposures, as in the present study, give an indication of acclimatisation capacity and are ecologically relevant to seasonal changes in salinity and carbonate chemistry, caution should be applied when extrapolating theses result to naturally assembled ecosystems. Lifelong and multigenerational responses to chronic changes in pCO_2 and salinity need further investigation. For example, reductions in feeding by the grazing mollusc *Littorina littorea* in response to elevated pCO_2 and temperature are no longer observed after 5 months of acclimation (Russell *et al* 2013). The relationship between energy available for growth and growth rate is complex. For example, *C. intestinalis* showed a loss in biomass in the ambient pCO_2 reduced salinity treatment despite the maintenance of SfG. This disconnect between energy available for growth and actual growth is likely to be due to changes in the carbon richness (i.e. energetic density/storage) of the tissues, the length of exposure to adverse conditions, and possibly changes in metabolic efficiency associated with body size.

Changes in carbonate chemistry and salinity may interact resulting in a variety of feeding and metabolic responses, effecting energy acquisition and utilisation that in-turn determines productivity. Interestingly, under natural feeding conditions, energy available for production is more dependent on feeding plasticity (i.e. the ability to regulate clearance rate and absorption efficiency) in response to elevated pCO_2 and reduced salinity than on changes in routine metabolic rates. This dependence on feeding plasticity shows the importance of understanding feeding plasticity, in addition to more commonly studied metabolic rates, in determining the comparative acclimatisation capacity of competing species to future climate change.

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955 **Tables:**

956 **Table 1.** Physico-chemical seawater measurements from each of the six nominal pCO_2 and salinity treatments over the 26 day exposure period

Nominal pCO_2 treatment (µatm)	500	500	500	1000	1000	1000
Nominal salinity treatment	30	23	16	30	23	16
pCO_2 treatment (µatm)	602±17.9^{A}	548±21.8^{A}	611±14.1^{A}	1045±54.0^{B}	964±19.2^{B}	1054±24.4^{B}
Salinity	30.5±0.11^{A}	22.9±0.22^{B}	16.2±0.15^{C}	30.3±0.17^{A}	22.9±0.39^{A}	15.6±0.16^{A}
Temperature (°C)	10.5±0.20^{A}	10.1±0.23^{A}	9.7±0.26^{A}	11.0±0.47^{A}	10.6±0.33^{A}	10.3±0.41^{A}
TA (µmol kg^{-1})	2206±7^{A}	1678±81^{B}	1227±129^{C}	2201±9^{A}	1625±24^{B}	1161±26^{C}
pH	7.88±0.01^{A}	7.84±0.02^{A}	7.67±0.01^{B}	7.67±0.01^{B}	7.60±0.02^{C}	7.43±0.01^{D}
DIC (µmol kg^{-1})	2105±5.10^{A}	1624±5.19^{B}	1226±1.31^{C}	2162±3.93^{D}	1618±29.7^{B}	1194±1.42^{C}
HCO_3^- (µmol kg^{-1})	1989±6.24^{A}	1546±6.81^{B}	1172±0.93^{C}	2060±3.55^{D}	1553±1.94^{B}	1131±0.75^{E}
CO_3^{2-} (µmol kg^{-1})	87.8±2.53^{A}	52.5±2.69^{B}	21.6±0.37^{C}	56.9±1.48^{B}	28.8±0.81^{D}	11.7±0.29^{E}
Ω_{calc}	2.15±0.61^{A}	1.33±0.07^{B}	0.57±0.01^{C}	1.40±0.04^{B}	0.74±0.02^{C}	0.31±0.01^{D}
Ω_{arag}	1.35±0.04^{A}	0.82±0.04^{B}	0.33±0.01^{C}	0.88±0.03^{B}	0.45±0.01^{D}	0.18±0.01^{E}
POM (mg L^{-1})	1.29±0.05^{A}	1.25±0.05^{A}	0.97±0.14^{A}	1.04±0.05^{A}	0.87±0.05^{A}	0.74±0.15^{A}
Suspended Particles 10ml^{-1} 1-1.5µm	26890±3230^{A}	21189±2327^{A}	25700±3926^{A}	28039±2465^{A}	25069±3026^{A}	26594±3257^{A}
Suspended Particles 10ml^{-1} 1.5-2µm	4944±163^{A}	4544±167^{A}	3683±131^{A}	6051±372^{A}	5430±345^{A}	4439±241^{A}
Suspended Particles 10ml^{-1} 2-2.5µm	2064±84^{A}	1925±106^{A}	1524±69^{A}	2468±165^{A}	2181±145^{A}	1748±97^{A}
Suspended Particles 10ml^{-1} 2.5-3µm	1033±42^{A}	982±57^{A}	796±53^{A}	1209±87^{A}	1072±66^{A}	865±57^{A}
Suspended Particles 10ml^{-1} 3-4µm	1334±59^{A}	1279±88^{A}	1087±114^{A}	1507±119^{A}	1332±82^{A}	1116±80^{A}
Suspended Particles 10ml^{-1} 4-5µm	1365±49^{A}	1301±84^{A}	1102±74^{A}	1560±142^{A}	1388±88^{A}	1183±111^{A}
Suspended Particles 10ml^{-1} 5-6µm	630±23^{A}	588±23^{A}	466±26^{A}	730±82^{A}	646±52^{A}	555±67^{A}
Suspended Particles 10ml^{-1} 6-7µm	371±13^{A}	356±15^{A}	266±15^{A}	425±51^{A}	364±28^{A}	302±32^{A}
Suspended Particles 10ml^{-1} 7-8µm	263±12^{A}	244±8^{A}	196±13^{A}	313±40^{A}	269±22^{A}	210±21^{A}
Suspended Particles 10ml^{-1} 8-9µm	178±7^{A}	164±6^{A}	125±7^{A}	209±25^{A}	181±16^{A}	129±11^{A}
Suspended Particles 10ml^{-1} 9-10µm	123±5^{A}	109±4^{A}	86±6^{A}	143±15^{A}	126±12^{A}	88±7^{A}

957 Temperature, salinity and pH (NBS scale) were measured 3 times daily. Total alkalinity (TA) was measured twice weekly. All other parameters [pCO_2; DIC (total dissolved inorganic carbon);
958 calcite and aragonite saturation state (Ω_{calc} and Ω_{arag}, respectively); HCO_3^-; and CO_3^{2-}] were calculated from pH and A_T with CO2SYS (Lewis and Wallace, 1998) using the dissociation after
959 Dickson and Millero (1987). Particulate organic matter (POM) is a mean across species at the time of CR determination. Concentration of suspended particles, within 10 size-intervals between 1
960 and 10µm in diameter, were determined every 48 h during the 26-day incubation using a laser particle counter (PAMAS GmbH, Model S4031GO), values are presented as the mean number of
961 particles $10ml^{-1}$ of seawater. Values are means ± s.e.m. Different superscript letters indicate significant variation between treatments (ANOVA, Tukey HSD post hoc, $P<0.05$).

962 **Table 2.** Estimated energetic parameters for surviving *C. intestinalis* after 26 days exposure to combined elevated pCO_2 and reduced salinity
963 treatments.

Nominal pCO_2 treatment (µatm)	500	500	1000	1000
Nominal salinity treatment	30	23	30	23
Energy Ingested ($J\ h^{-1}$)	34.9±3.41 ^{A}	40.0±3.05 ^{A}	19.4±2.97 $^{1\,B}$	5.7±3.21 $^{2\,B}$
Absorption Efficiency (%)	34.8±7.51	39.2±3.78	23.4±1.49	27.5±1.62
Energy Absorbed ($J\ h^{-1}$)	14.8±2.39 ^{A}	15.3±2.28 ^{A}	1.1±2.39 ^{B}	5.1±2.22 ^{B}
Scope for Growth ($J\ h^{-1}$)	14.7±2.64 ^{A}	15.2±2.28 ^{A}	4.9±2.22 ^{B}	1.0±2.39 ^{B}

964
965 Values are estimated means ± s.e.m generated from the GLMM (pCO_2*Salinity) and adjusted to the mean mass of sampled individuals (120.1 mg DW). Different superscript
966 numbers and letters indicate significant variation ($p > 0.05$) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means.
967 For Absorption Efficiency values are mean % ± s.e.m with statistical comparisons as above but based on arc sign square root transformed data. Numbers indicate significant
968 effects of salinity within each level of pCO_2. Letters indicate significant effects of pCO_2 within each level of salinity.
969

970 **Table 3.** Estimated energetic parameters for *M. edulis* after 26 days exposure to combined elevated pCO_2 and reduced salinity treatments.

Nominal pCO_2 treatment (µatm)	500	500	500	1000	1000	1000
Nominal salinity treatment	30	23	16	30	23	16
Energy Ingested (J h^{-1})	68.7±4.64 ^{1}	58.3±4.38 ^{1}	21.5±4.37 $^{2\,A}$	62.5±4.37 ^{1,2}	63.8±4.67 ^{1}	48.1±4.36 $^{2\,B}$
Absorption Efficiency (%)	32.8±2.34 $^{1\,A}$	47.5±1.07 $^{2\,A}$	48.1±0.99 $^{2\,A}$	19.5±0.93 $^{1\,B}$	31.3±0.79 $^{1,2\,B}$	44.6±1.58 $^{2\,B}$
Energy Absorbed (J h^{-1})	22.8±1.79 $^{1\,A}$	27.7±1.70 $^{2\,A}$	10.3±1.70 $^{3\,A}$	12.1±1.70 ^{B}	14.3±1.80 ^{B}	15.8±1.70 ^{B}
Scope for Growth (J h^{-1})	22.2±1.82 $^{1\,A}$	27.3±1.72 $^{2\,A}$	9.8±1.72^{3}	10.7±1.72^{B}	13.4±1.84^{B}	15.0±1.82

971

972 Values are estimated means ± s.e.m generated from the GLMM (pCO_2*Salinity) and adjusted to the mean mass of sampled individuals (598.5 mg DW). Different
973 superscript numbers and letters indicate significant variation (p >0.05) established by F-tests based on linearly independent pairwise comparisons among the estimated
974 marginal means. For Absorption Efficiency values are mean % ± s.e.m with statistical comparisons as above but based on arc sign square root transformed data. Numbers
975 indicate significant effects of salinity within each level of pCO_2. Letters indicate significant effects of pCO_2 within each level of salinity.

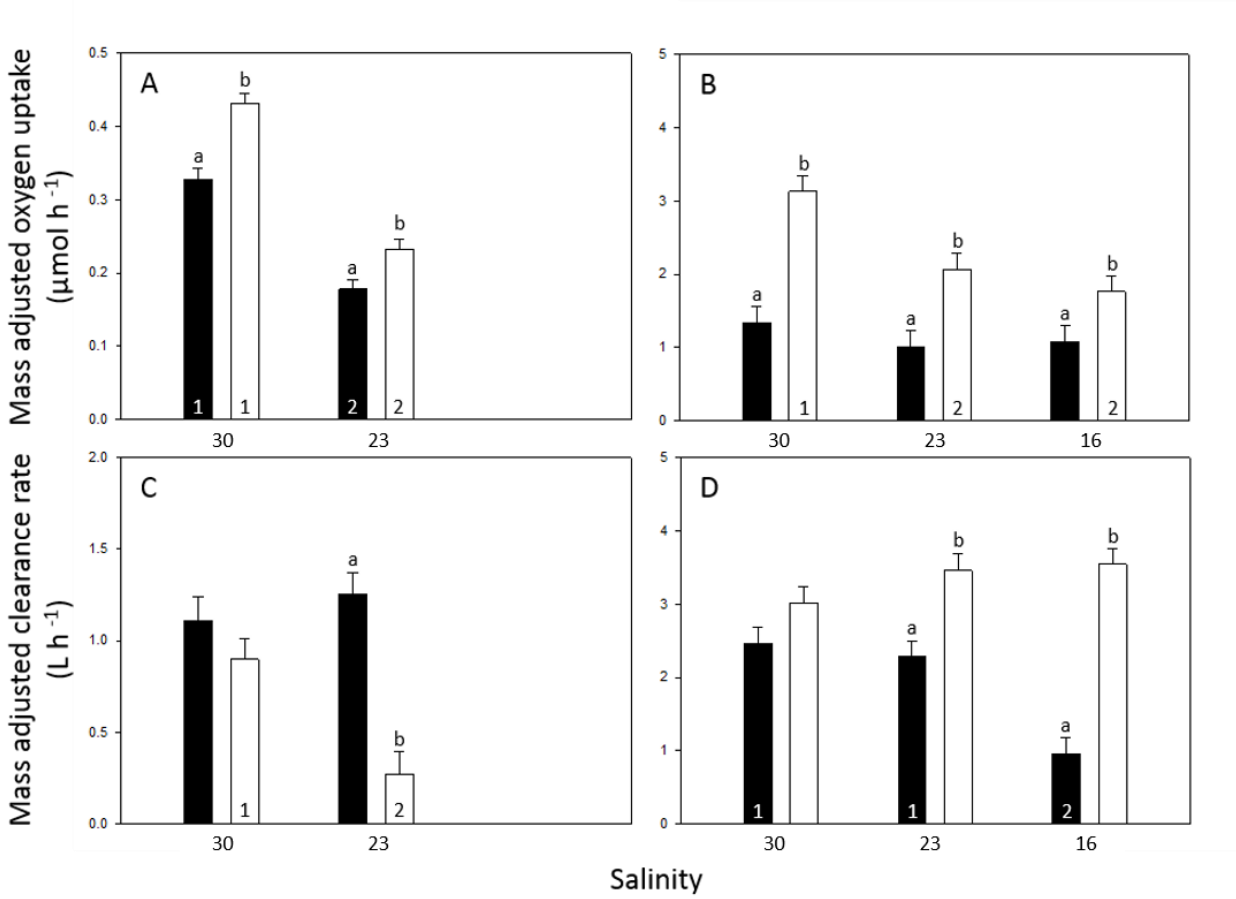
976

Figure legends:

Figure 1. Oxygen uptake and Clearance rate in *C. intestinalis* (A and C respectively) after 26 days exposure to 23 or 30 salinity and *M. edulis* (B and D respectively), after 26 days exposure to 16, 23 or 30 salinity at ambient (500 µatm; black bars) or elevated (1000 µatm; white bars) pCO_2. No data is shown for *C. intestinalis* at 16 salinity due to 100% mortality in this treatment. Values are estimated means ± s.e.m generated from the GLMM (pCO_2*Salinity) and adjusted to the mean mass of sampled individuals (*C. intestinalis* = 120.1 mg DW; *M. edulis* = 598.5 mg DW). Different numbers and letters indicate significant variation ($p > 0.05$) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means. Numbers indicate significant effects of salinity within each level of pCO_2. Letters indicate significant effects of pCO_2 within each level of salinity.

Figure 2. SfG, AFDW:DW and Growth rate for *C. intestinalis* (A, C and E respectively) after 26 days exposure to 23 or 30 salinity and *M. edulis* (B, D and F respectively) after 26 days exposure to 16, 23 or 30 salinity at ambient (500 µatm; black bars) or elevated (1000 µatm; white bars) pCO_2. No data is shown for *C. intestinalis* at 16 salinity due to 100% mortality in this treatment. Values are estimated means ± s.e.m generated from the GLMM (pCO_2*Salinity) and adjusted to the mean mass of sampled individuals (*C. intestinalis* = 120.1 mg DW; *M. edulis* = 598.5 mg DW). Different numbers and letters indicate significant variation ($p > 0.05$) established by F-tests based on linearly independent pairwise comparisons among the estimated marginal means Numbers indicate significant effects of salinity within each level of pCO_2. Letters indicate significant effects of pCO_2 within each level of salinity.

1011 **Figure 1:**



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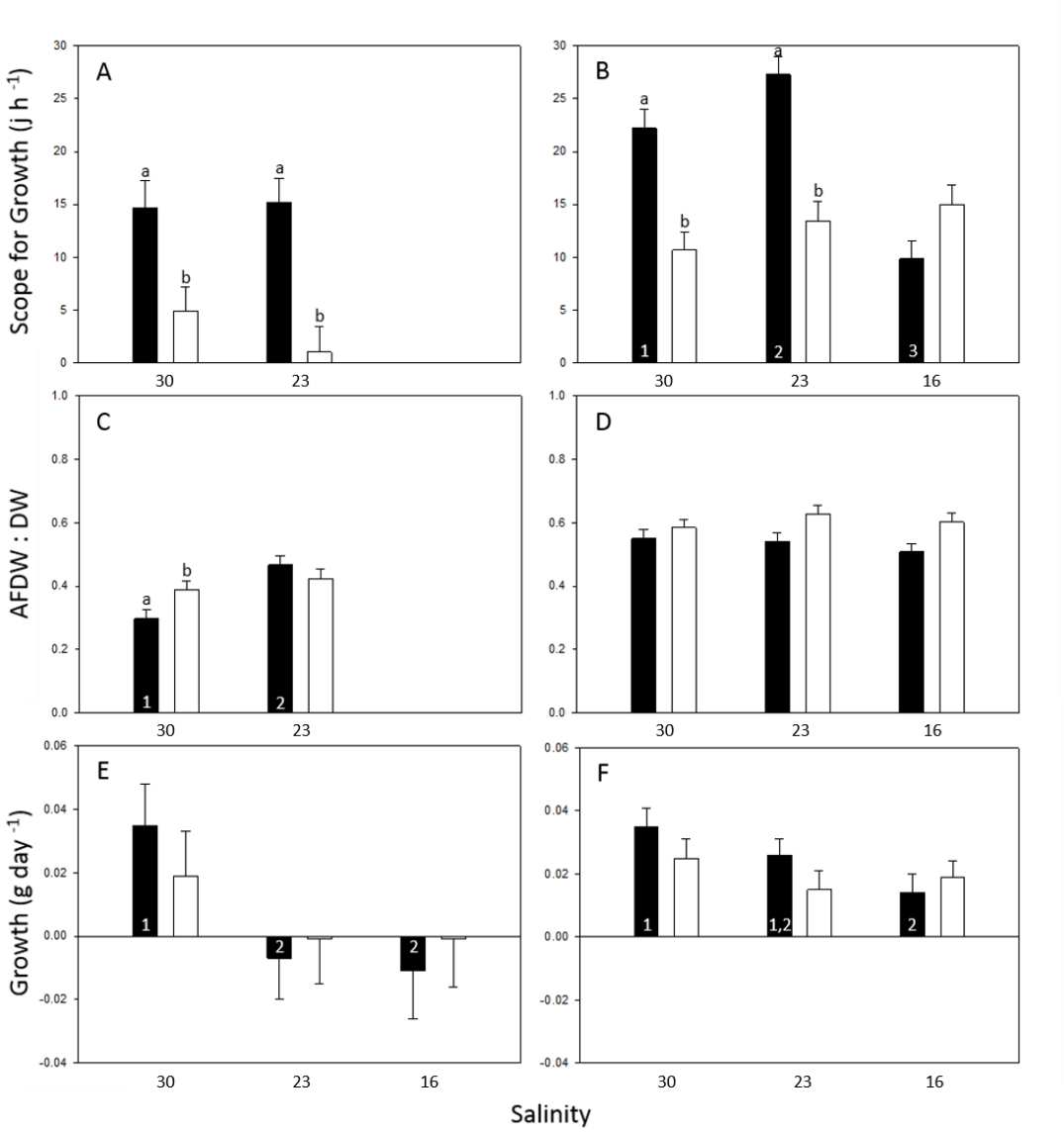
1022 **Figure 2**



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